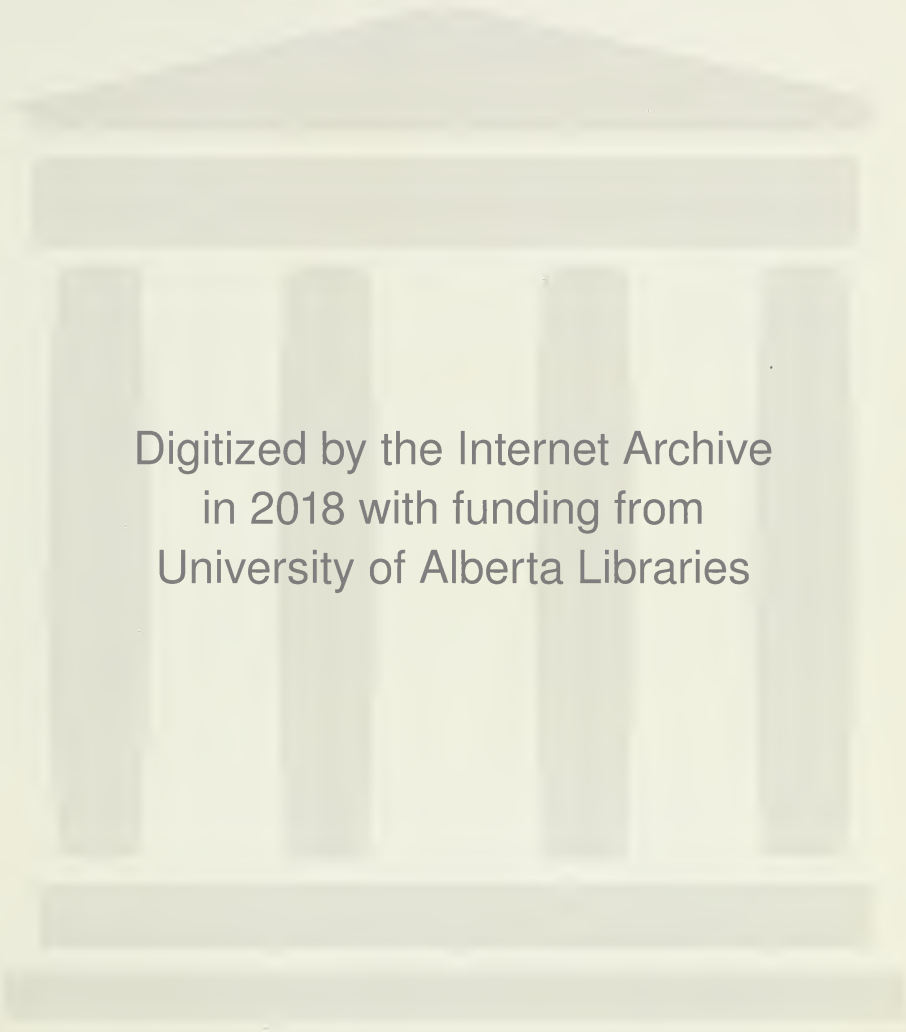
I. ON THE GENESIS OF HEMOGLOBIN IN THE INCUBATED HEN EGG

and

II. ON THE SYNTHESIS OF ERGOTHIONEINE

by

MARGARET SHIRLEY FRASER
B.Sc. (Alberta)



THE UNIVERSITY OF ALBERTA

I - ON THE GENESIS OF HEMOGLOBIN IN THE INCUBATED HEN EGG

II - ON THE SYNTHESIS OF ERGOTHIONEINE

A DISSERTATION

SUBMITTED TO THE SCHOOL OF GRADUATE STUDIES

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE

OF MASTER OF SCIENCE

FACULTY OF ARTS AND SCIENCE

BY

MARGARET SHIRLEY FRASER

EDMONTON, ALBERTA

APRIL, 1950

ACKNOWLEDGEMENT

I wish to thank Dr. G. Hunter for his helpful advice and criticism and Dr. J. Tuba for his guidance and assistance throughout the course of this investigation.

My thanks are due also to the other members of the Biochemistry and Chemistry Departments who have helped me with their suggestions.

I am also indebted to Dr. R. Fraser for performing the nephrectomies and injecting the rats; to Mr. R. Clelland for his help with the experimental animals; and to Mr. P. Beamont for his willing, general assistance.

This work was made possible through a Medical Research Grant, part of M.P. 189, from the National Research Council.

ON THE GENESIS OF HEMOGLOBIN IN THE INCUBATED HEN EGG

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PREFACE

Hans Fischer (6) in 1929 completed a chemical synthesis of hematin, the iron complex of protoporphyrin, and succeeded in combining this in its reduced form with the protein globin, thereby producing hemoglobin, which was similar in all respects to the natural blood pigment. However, the complete mechanism of the synthesis of hemoglobin in the animal body is still unknown.

A study of the iron and copper metabolism in embryonic life would be one way of approaching the problem of the biosynthesis of hemoglobin and the developing chick embryo was chosen for this purpose. Any information gained from such an investigation would add to the existing knowledge of hemoglobin synthesis which is presented, in summary, in this introduction.

The pigments mentioned here may be regarded as being derived from porphin, a substance consisting of four nitrogen-containing pyrrole units joined in a ring structure by means of four methine groups (Fig. 1).

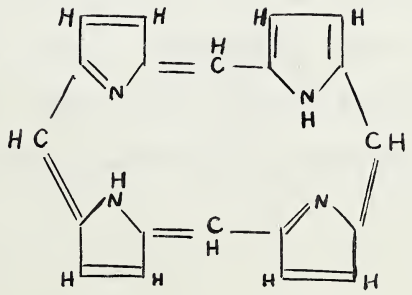


Fig. 1 - Porphin

Each pyrrole group of porphin posesses two hydrogen atoms which may be replaced by other groups, for example, in aetioporphyrin there are four methyl groups and four ethyl groups attached at the eight corners of these rings in place of the hydrogens of porphin. This substitution may be made in different ways, resulting in four possible different aetioporphyrins which Fischer has named aetioporphyrin I, II, III, and IV, and each is capable of giving rise to a potential series of porphyrin derivatives (Fig. 2).

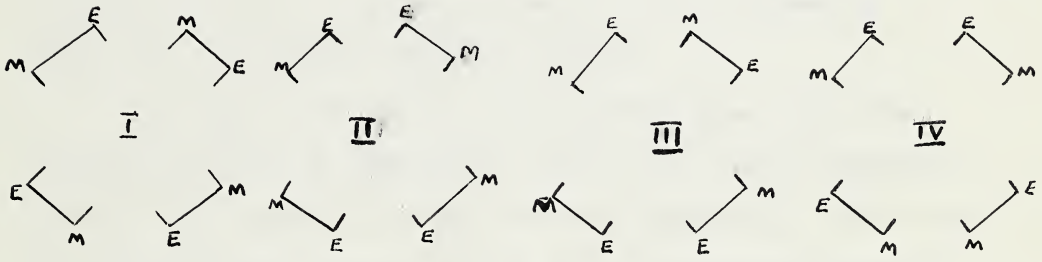


Fig. 2 - The Four Aetioporphyrins (⌐¬ = pyrrole ring).

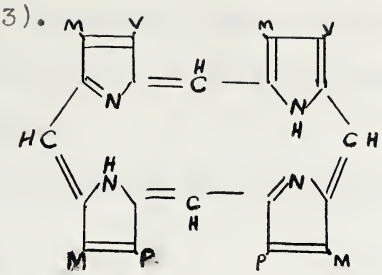
Only two of these four possible kinds of porphyrins are known in nature, derivable from aetioporphyrin I and III respectively.

Most of the pigments such as hemoglobin, myoglobin, cytochrome, catalase, bilirubin, urobilin, chlorophyll, etc., belong to series III, while series I isomers are excreted in large quantities in certain pathological conditions.

In most animals the iron necessary for hemoglobin synthesis is furnished by ferritin, the brown iron-containing protein present in spleen, bone marrow, and liver. The iron in ferritin is stored in the form of colloidal micelles of very insoluble basic iron phosphate attached to a large, non-diffusible protein molecule, apoferritin (7). When iron is required for heme synthesis, it is made rapidly available , probably by reduction of the ferritin iron to the ferrous form. Demands for globin synthesis may be met by breaking down the apoferritin molecule itself.

The iron is believed to be incorporated into the prosthetic part of hemoglobin after the cyclic structure of the four pyrrole groups has been completed. This view is supported by certain facts: 1. H. Influenza requires heme for growth (8). When grown on certain iron-free porphyrins, such as protoporphyrin, these organisms insert the iron and their growth requirements are thus met. The vinyl groups seem to be necessary to make possible this introduction of iron into the ring. 2. Protoporphyrin occurs in immature erythrocytes which are rapidly synthesizing heme (4). Protoporphyrin, then, not some iron-containing open-chained tetrapyrrole, is considered to be the precursor of hemoglobin. Its constitution is as follows and it can be seen that it is derived from aetioporphyrin

Type III (Fig. 3).



M = $-CH_3$
V = $-CH=CH_2$
P = $-CH_2CH_2COOH$

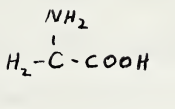
Fig. 3 - Protoporphyrin

Rimington (21) suggests that pyrrole precursors (probably dipyrrylmethenes) are capable of combining to give either series I or III pigments, but that the synthesis is selectively catalyzed so that the latter predominate. The small amounts of coproporphyrin I which are excreted in normal urine may be considered as a by-product in the synthesis of Type III pigments.

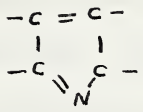
The first step in the porphyrin synthesis suggested by the discovery by Shemin and Rittenberg (24), is that glycine is the nitrogenous precursor of the pyrrole ring. Also acetic acid or a closely related compound appears to take part in the synthesis. More recent discoveries by Muir and Neuberger (18) show that the acidic and non-acidic pyrrole nuclei in hemin have the same or closely similar isotope ratios, i.e., there is an equal distribution of N^{15} over the four rings of protoporphyrin. By using glycine labeled with C^{14} on the

carboxyl group, Grinstein, Kamen and Moore (9) showed that this group in glycine does not appear in the protoporphyrin molecule.

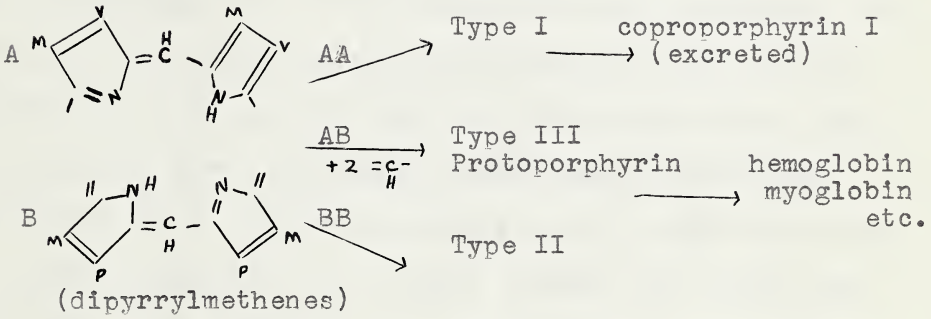
The present theories may be summarized as follows:



(glycine)



(pyrrole)



(dipyrrylmethenes)

V = $-CH=CH_2$

P = $-CH_2CH_2COOH$

M = $-CH_3$

Copper plays an important role in this synthesis but its mechanism of action is, as yet, unknown.

The iron of the hen egg is nearly all located in the yolk where it is combined with a material which has certain protein-like properties. Bunge (2) studied this and named it haematogen. Hugounenq and Morel (11) regarded haematogen as being the precursor of the hemoglobin formed by the chick during the incubation of the egg. Support is lent to this theory by the fact that coincident with the great increase in the hemoglobin content of the chick on the fourteenth day of incubation as shown by Sendju (23), there is a corresponding sudden decrease in the vitellin-phosphorus of the yolk as shown by Plimmer and Scott (20). Hugounenq and Morel decomposed haematogen by acids and split off a black iron-containing pigment (2.60% Fe) which they named haematovin. McFarlane, Fulmer, and Jukes (17) state that it is probable that the synthesis of hemoglobin has been partly completed in the body of the hen before the egg is laid, the embryo completing the formation of hemoglobin from this preformed compound.

The subject of this investigation was originally intended to be this so-called "preformed precursor", in order to determine whether the mode of synthesis of hemoglobin in the developing chick was similar to that which has been summarized above.

A review of the literature showed that there is considerable controversy as to whether the iron is inorganic, or organically bound in a fraction in vitellin which serves as an intermediate in the hemoglobin production in the chick. Therefore the present work was reduced to a consideration of the state of iron and copper in the egg.

SECTION I - THE STATE OF IRON IN THE EGG.

INTRODUCTION

In the earlier literature Bunge applied the term, haematogen, to iron-containing fractions obtained from the breakdown of the vitellin molecule. Hill (10), using the α,α'-dipyridyl and sodium hydrosulfite method for iron determinations, concluded that iron was present as colloidal ferric hydroxide and that yolk contained no iron in a strong organic combination such as hematin.

McFarlane (16) hydrolyzed lecitho-vitellin with 0.25 N sodium hydroxide and obtained a fraction, not precipitable with trichloroacetic acid but precipitable with normal lead acetate, which contained all the iron and 25% of the copper of the material hydrolyzed. The metals, present in the filtrate after decomposition

of the lead acetate precipitate with hydrogen sulfide, did not give direct reactions with potassium thiocyanate or sodium diethyldithiocarbamate. Hence he concluded that iron and copper were in organic form as part of the vitellin molecule.

McFarlane's views are not supported by Tompsett (26) who used thioglycolic acid for estimation of iron. In trichloroacetic acid filtrates of egg yolk, and of egg yolk plus iron alum, negative tests resulted with thioglycolic acid and ammonium hydroxide, although iron added to the filtrate could be determined, thus showing that there is nothing which interferes with the test. If the reaction with thioglycolic acid is carried out before addition of the trichloroacetic acid, the iron in egg is readily estimated quantitatively in the filtrate and ferric iron added to the yolk can be recovered.

However, the work of Shorland and Wall (25), confirmed by Borgen and Elvehjem (1) showed that thioglycolic acid and sodium hydrosulfite react with traces of hematin and hemoglobin to release iron. It is not inconceivable then that if the iron were in weak organic combination in the yolk, sodium

hydrosulfite might split it off to give Hill's positive α,α'-dipyridyl test; and thioglycolic acid might release iron to give Tompsett's test when this reagent was used before precipitation with trichloroacetic acid. Kitzes, Elvehjem, and Schuette (12) reported that iron was co-precipitated with plasma proteins when trichloroacetic acid was used. They believed the iron was mechanically carried down. This might explain why Tompsett obtained no positive test with iron added to egg yolk.

More information was needed to decide whether iron was loosely combined with the protein or was actually in organic combination in vitellin. It was decided to add inorganic iron to egg yolks and to see whether this would appear in the vitellin prepared from these. If the iron forms a complex, its content in the vitellin will have increased. Under these conditions, provided the iron originally present in the egg is in some sort of organic combination, then by repeating McFarlane's hydrolysis with this vitellin, the difference between the two types of iron might be manifested in the separate hydrolytic fractions.

RESULTS AND DISCUSSION

Determination of Iron

A. Iron was determined according to the method of Kitzes et al (12) with certain modifications. The above method was developed for the estimation of plasma iron of 0-5 µgm. range. Determination of iron in more concentrated solutions is attended with certain difficulties, noted below, which the modifications introduced correct.

B. The digestion procedure was essentially that of McFarlane (15).

A. Preparation of the Standard Graph.

Reagents

1. Buffer - 12.5 gm. sodium acetate plus 12.5 ml. glacial acetic acid were diluted to 100 ml. (PH 4.7).
2. α,α'-dipyridyl - 0.2% solution in 0.1 N HCl.
3. Thioglycolic acid - Eastman grade, redistilled.
4. Iron standard solutions containing 50 µgm. Fe/ml.

   a. 0.3511 gm. pure salt, $FeSO_4(NH_4)_2SO_4.6H_2O$ are dissolved in a little water and 100 ml. 1.0 N HCl and diluted to 1 liter.

   b. 0.0500 gm. analytical iron wire are dissolved in 17 ml. 6 N HCl and diluted to 1 liter. The working standards containing 5 µgm. Fe/ml. were prepared by diluting 10 ml. of each of these solutions to 100 ml.

The HCl used was double distilled. Glass-distilled water was used to prepare the standard iron solutions.

Procedure

Standard colored solutions of 10 ml. volume in colorimeter tubes were prepared in the range 5 - 25 μgm. Fe as follows:

X ml. standard Fe solution containing 5 μgm. Fe/ml.

3.0 ml. buffer solution.

7-X ml. glass distilled water.

Two drops of thioglycolic acid were added to each tube and the tubes placed in a water bath at 60° C for 5 minutes. The tubes were allowed to cool in a beaker of water without shaking.

The center settings were then determined on the Evelyn colorimeter, using filter 520 mμ, by setting each tube in the holder, adjusting the galvanometer to read 100, removing the tube and noting the reading. Thus each tube serves as its own blank, with the blank tube measuring the amount of Fe contamination in the reagents.

Then 1 ml. 0.2% α,α'-dipyridyl solution was added and the color development measured immediately using the previous center setting for each tube. L values (L= 2-log G) were calculated from the galvanometer

readings (G) and $L_{aliquot} - L_{blank}$ values were plotted against µgm. Fe. These values were checked using the standard Fe solution prepared from analytical iron wire.

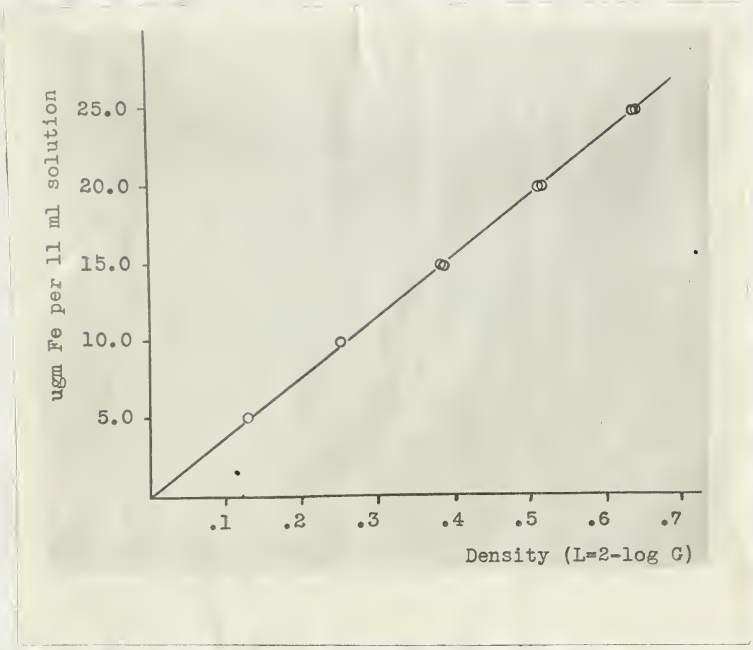


FIGURE 3 - IRON CALIBRATION GRAPH.

B. Digestion

The materials for iron analyses were transferred to micro-Kjeldahl flasks. One ml. concentrated sulfuric acid, 0.5 ml. 60% perchloric acid, and a glass bead were added and the digestion carried out with the flasks placed in a sand bath. Heating was continued until dense white fumes formed. The flasks were cooled and one drop of concentrated nitric acid was added and the digest heated again until the excess nitric acid was driven off.

The digest was cooled, transferred to a 10 ml. volumetric flask with three one ml. portions of water and then 2.5 ml. 40% NaOH was added carefully. This was made to volume after cooling, and iron determinations were carried out according to the above method with a 2 or 1 ml. aliquot. The amount of Fe in the test solution was calculated from the calibration graph or from the formula $(L_{aliquot} - L_{blank}) \times 39.3$, where 39.3 is the slope of the graph.

Modification of the method of determining iron.

Center settings obtained with tubes containing a fair amount of iron, say 25 µgm., were found to be 4 or 5 galvanometer readings greater than the center settings of the blanks. If the reduction of the iron were allowed to proceed for about an hour, the center

settings decreased until they approximated those of the blanks. Findings in a typical experiment reported in Table I show this effect.

TABLE I - Effect of time of reduction on center settings.

Tube number	1	2	3	4	5	6 (Blank)
Fe content (μgm.)	5	10	15	20	25	0
Center settings						
at 10 mins.	79^{2}	80	82	82^{2}	83^{2}	$79'$
15 "	79^{2}	79^{3}	$81'$	$82'$	$83'$	79
30 "	$79'$	79^{3}	79^{3}	80^{3}	81^{2}	$79'$
65 "	$79'$	79^{2}	79^{2}	$79'$	80	79
100 "	79	79^{2}	$79'$	79	79^{2}	79

It was found that heating the tubes for 5 minutes at 60°C, after adding the thioglycolic acid, hastened reduction and produced the same effect on the center settings as allowing the tubes to stand at room temperature for about an hour.

When α,α'-dipyridyl was added after the tubes had been heated and cooled, full color development was attained immediately. By the method of Kitzes et al,

full color was not attained for about an half hour or longer, due presumably, to the fact that not all the ferric iron was reduced to the ferrous form to react with the α,α'-dipyridyl to produce the colored complex.

A transient blue color is formed with ferric iron and thioglycolic acid in acid solution (13). As the iron is gradually reduced by the thioglycolic acid, the color fades. Therefore, at minimum color (i.e., when the center setting has fallen to its lowest value after 5 minutes heating), all the iron may be assumed to be in the ferrous form. Addition of α,α'-dipyridyl at this point results in full color development..Adding the dipyridyl when the center setting has a higher value, because of the presence of ferric iron, results in an erroneous final galvanometer reading.

The above modification eliminates this error and reduces the time of reduction and final color development.

Preparation of vitellin (3).

The yolks of six eggs were separated from the whites and washed thoroughly with running water and 0.9% saline without rupturing the yolk membrane. The yolks were then broken into an equal volume of 10% saline solution and extracted with ether containing 2% ethyl alcohol until the ether extracts were colorless. The water solution was strained through cheesecloth to remove the yolk membranes. The solution was then poured into 20 volumes of water and allowed to stand overnight. The supernatant was siphoned off and the remaining precipitate and fluid were centrifuged. Without washing, the precipitate was dissolved in 10% saline and again precipitated by pouring into 20 volumes of water. The material was collected as before, washed once with water, and centrifuged again.

The protein was suspended in 1200 ml. 80% ethanol and refluxed for several hours. The precipitate was filtered as dry as possible by suction, and the process repeated using 95% and then absolute ethanol. It was filtered and washed with ether and dried in a vacuum dessicator over sulfuric acid for several days. The yield obtained was 7 gm. An analysis for iron and copper gave 0.051% Fe, and 0.0039% Cu.

Preparation of vitellin with increased iron content.

Elvehjem and Hart (5) state that the iron content of an egg of average size is about 0.8 - 1.0 mgm. The average vitellin content per egg is approximately 2 gm., so this results in an iron concentration in vitellin of about 0.04 - 0.05%.(16). This percentage of iron in vitellin can be considered fairly constant since Schaible, Davidson,and Bandener(22) have shown that there is no significant difference in iron contents of yolks of eggs produced before or after iron supplementation to the diet of the hen.

To the saline solution of two egg yolks, 2900 µgm. iron as ferric chloride was added. The mixture was extracted with ether and the vitellin prepared as above. % Fe found - 0.147. The increase from about 0.05 to 0.147% was then apparently due to the inorganic iron which appeared almost quantitatively in the vitellin, as shown by the following approximations.

Vitellin content from two eggs	4 gm.
Approximate Fe content	2000 µgm.
Added Fe	2900 µgm.
% Fe (calculated)	0.122
% Fe (found)	0.147

Hydrolysis of vitellin.

To 0.9279 gm. vitellin (0.051% Fe) in a 150 ml. Erlenmeyer flask was added 100 ml. 0.25N sodium hydroxide. The flask was placed in a thermostat at 37°C for 72 hours.

a. 15 ml. of the digest was slowly added to 25 ml. of 3.5% trichloroacetic acid in a centrifuge tube. The supernatant was drained off completely from the resulting precipitate and iron analyses were done on the whole of the precipitate and on aliquots of the supernatant. Iron contamination of the precipitate due to the small amount of adhering supernatant would be negligible since 1 ml. of the supernatant contains less than 2 µgm. Fe.

b. To 25 ml. of this supernatant was added 5 ml. of saturated normal lead acetate in a centrifuge tube. The supernatant was drained off and iron determinations were done on the whole of the lead acetate precipitate and on aliquots of the supernatant.

A second 15 ml. aliquct of this digest was treated in the same manner except with the addition of 25 µgm. Fe to the trichloroacetic acid filtrate before lead acetate precipitation.

Similar hydrolysis experiments were carried out with the vitellin containing 0.147% Fe. The findings are given in Table 1.

Table (2) - Hydrolysis of vitellin with 100 ml. 0.25N NaOH at 37 C for 72 hours.

Hydrolysis experiment		1	2	3	4
Weight sample (gm.)		0.9279	0.9279	0.6973	0.4019
% Fe		0.051	0.051	0.147	0.147
Fe in 100 ml. digest	(µgm.)	473.2	473.2	1025.0	590.8
Fe in 15 ml. digest (calcul'd)	''	70.9	70.9	153.8	88.6
Fe in trichloroacetic acid precipitate	''	13.5		86.5	
Fe in 40 ml. supernatant	''	57.6		64.0	
Fe in 25 ml. supernatant (calcul'd.)	''	28.5	425.0	40.0	
Fe in Pb acetate precipitate	''	19.9	29.9	35.3	30.4
Fe in Pb acetate supernatant	''	14.0			14.8

From the hydrolysis experiment it was found that the lead acetate precipitate did not account for all the iron. Some iron was precipitated by trichloroacetic acid and some remained in the lead acetate filtrate. Iron added to the trichloroacetic acid filtrate in experiment 2 could be precipitated by lead acetate, although McFarlane found this not to be the case, even though his trichloroacetic acid filtrate contained large amounts of iron due to contamination from the flask.

Therefore, McFarlane's statement that all the iron was contained in the lead acetate precipitate could not be confirmed. It appears also from these experiments that the lead acetate precipitate shows no distinction between the iron added to vitellin and that originally present in the yolk.

When McFarlane decomposed the lead acetate precipitate with hydrogen sulfide, iron and copper were found in the filtrate. This was the basis for his conclusion that iron and copper were organically bound. Recently Neuberg (19) has reported that copper and iron sulfides are soluble in solutions of certain amino acids and proteins. This could explain McFarlane's findings.

SECTION II - THE STATE OF COPPER IN THE EGG.

INTRODUCTION

Copper has been reported to be part of the vitellin molecule. McFarlane (16) places the value at 0.0033%, and states that it is not improbable that all the copper of the yolk is contained in this protein. Tompsett (27) performed recovery experiments with copper added to various biological materials. Copper was added to egg yolk and other materials and then determined in the trichloroacetic acid filtrates, allowances being made for the initial copper contents of the materials. Since it was recovered quantitatively from yolks, Tompsett concluded that copper does not form a complex as does iron. This statement along with the fact that vitellin contains copper would appear to confirm McFarlane's belief that copper in vitellin is organically bound.

Since the copper originally present in the egg yolk appeared in the trichloroacetic acid filtrate, one can conclude from Tompsett's experiments that either the trichloroacetic acid split the copper from its organic combination, or that copper does form a complex which can be split by trichloroacetic acid.

Therefore it was decided to add inorganic copper to the yolk and ascertain whether it would appear in the vitellin prepared from this yolk. Increased copper content would indicate formation of a complex.

RESULTS AND DISCUSSION

Determination of copper - The sodium diethyldithiocarbamate method was used as described by McFarlane (14).

Reagents

1. Sodium pyrophosphate solution - 4% $Na_4P_2O_7.10H_2O$.
2. Concentrated ammonium hydroxide.
3. Redistilled amyl alcohol.
4. Sodium diethyldithiocarbamate solution - 0.5% in water.

Digestion

The destruction of organic matter by wet incineration was carried out in a micro Kjeldahl flask. Two ml. concentrated sulfuric acid and 1 ml. 60% perchloric acid were added and the solution was heated in a sand bath until white fumes ceased to be evolved.

Preparation of standard copper solution

0.3928 gm. pure $CuSO_4.5H_2O$ was dissolved in water with a small amount of dilute sulfuric acid. The solution was diluted to 1 liter (1 ml. = 0.10 mgm. Cu). The standard copper solution was prepared by diluting 10 ml. of the above solution to 100 ml. (1 ml. = 10 µgm. Cu).

Determination of copper

The contents from the Kjeldahl flask were transferred to a volumetric flask and diluted to volume. A 15 ml. aliquot was taken for analysis in a 50 ml. stoppered graduated cylinder. The standard was prepared in exactly

the same way except with the omission of the vitellin. Ten µgm. of the copper standard was added to the aliquot in the cylinder. Two ml. of the pyrophosphate solution was added and contents were made alkaline with concentrated ammonia. Then 10 ml. amyl alcohol and 0.5 ml. 0.5% sodium diethyldithiocarbamate were added and the cylinder was stoppered and shaken for 30 seconds. The alcohol layer was allowed to settle and after it was clear, 5 ml. was pipetted into clean cups in a Duboscq colorimeter and the colors were compared with the standard containing the 10 µgm. copper.

Preparation of vitellin.

Vitellin was prepared as above from one egg but with the addition of 120 µgm. copper as copper sulfate before the ether extraction. % Cu - 0.0086.

The average amount of copper in vitellin is said to be less than 0.004%.(16). The increase from about 0.004 to 0.0086% was then apparently due to the inorganic copper which appeared almost quantitatively in the vitellin, as shown by the following approximations.

Vitellin content from one egg	2 gm.
Approximate Cu content	80 µgm.
Added Cu	120 µgm.
% Cu (calculated)	0.010
% Cu (found)	0.0086

Experiments with copper in vitellin.

0.5543 gm. vitellin (0.0039% Cu) was suspended in 25 ml. trichloroacetic acid for about 3 hours at room temperature with occasional shaking. Then copper determinations were made on aliquots of the filtrate.

0.5543 gm. vitellin	
Calculated copper content	21.5 µgm.
Copper extracted with trichloroacetic acid	19.0 µgm.

From the above work, it can be concluded that Cu does form a type of complex with vitellin and is easily removed from its combination by extracting with 20% trichloroacetic acid. From similar experiments, it was found that iron in vitellin was not easily extracted by 20% trichloroacetic acid but that it could be released by 1.5N HCl. Iron appears to be more strongly held than copper to the vitellin protein.

SUMMARY

1. A modification of the method of Kitzes et al for iron determinations has been described.
2. Iron added to egg yolk appears in the vitellin prepared from these yolks.
3. It was concluded after repeating McFarlane's hydrolysis experiments that iron is not in organic combination with vitellin.
4. Tompsett's statement that copper does not form a complex with vitellin was not confirmed.
5. Copper added to egg yolk appears in the vitellin prepared from these yolks. McFarlane's claim that copper is in organic combination with vitellin is refuted.

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ON THE SYNTHESIS OF ERGOTHIONEINE

Table of Contents

PREFACE

In 1909 Tanret (33) first isolated the base, ergothioneine, from ergot of rye. Hunter and Eagles (20), and Benedict, Newton, and Behre (5) isolated from pig blood sulfur-containing compounds which they named sympectothion and thiasine, respectively. It remained for Eagles and Johnson (8) to identify both products from pig blood as ergothioneine. The structural formula was established by the work of Barger and Ewins (4) in 1911, who showed ergothioneine to be the betaine of thiolhistidine (Fig. 1). But the ultimate proof for the structure of any compound rests on its chemical synthesis.

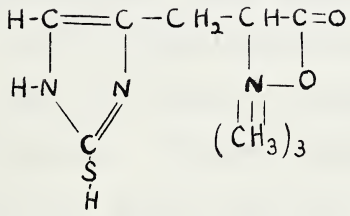


Fig. 1 - Ergothioneine.

No physiological significance was attached to ergothioneine, a constituent of red blood cells, until Lawson and Rimington (23) attempted to demonstrate an anti-thyroid effect with the compound. A series of papers on other aspects of ergothioneine have since been published as a result of renewed interest in ergothioneine research (Stanley and Astwood (32), Hunter (19), Work (35), Woolf (34), and Latner and Mowbray (22)). In order to carry out physiological

and clinical experiments, adequate supplies of ergothioneine are necessary and so the problem of the chemical synthesis has become more pressing. Part A of this work is concerned with the attempted chemical synthesis. The biochemical studies on ergothioneine described in Part B were undertaken in an attempt to explain its origin in the red blood cell and its role, if any, in the organism.

PART A - CHEMICAL SYNTHESIS

INTRODUCTION

In 1930, Ashley and Harington (3) attempted the synthesis of ergothioneine. They suggested that the hypothetical amino acid, 2-thiolhistidine, first introduced by Eagles and Vars (9), might be methylated in vivo to produce ergothioneine. However, methylation of this compound by chemical means resulted in a pentamethyl derivative. Again in 1933, Harington and Overhoff (17) reported more attempts at synthesis. Their methods are outlined below, followed in each case by a brief statement of their results.

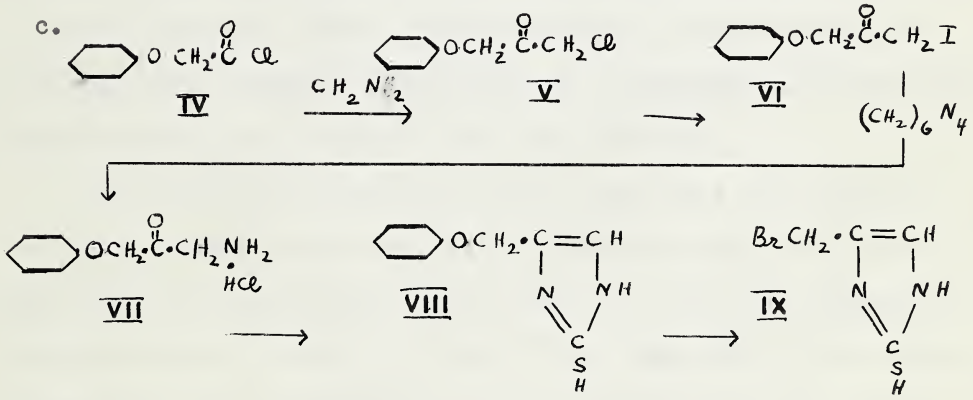
a. Benzoylate ethyl α-chloro-β-glyoxaline-4(or 5)-propionate (I). Convert this by controlled hydrolysis to α-chloro-γ-keto-δ-aminovaleric acid. Condense with potassium thiocyanate to form the α-halogeno-β-2-thiol-glyoxaline-4(or 5)-propionic acid.(II). Substitute the halogen by trimethylamine to produce ergothioneine.

Their attempts to benzoylate I failed. It appears that the presence of a negative halogen atom in the side chain of I renders the glyoxaline ring resistant to fission by benzoylation.

b. Convert the amino groups of the disulfide of 2-thiol-4(or 5)-aminomethylglyoxaline (III) to hydroxyls and finally replace by halogens. Condense with ethyl sodiochloromalonate, reduce the resulting disulfide, hydrolyze and decarboxylate, to give II.

The action of iodine to produce the disulfide from III did not proceed smoothly and a complex mixture was obtained.

c.



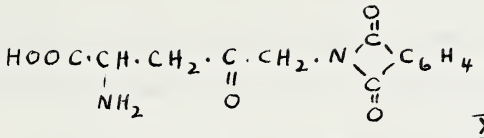
Compound VIII could not be satisfactorily hydrolyzed to 2-thiol-4(or 5)-hydroxymethylglyoxaline, nor could the phenoxy group be directly substituted by bromine. The object would be to obtain IX and condense it with ethyl sodiochloromalonate to produce II.

d. Condense phthalimidohalogenoacetone with ethyl sodiochloromalonate, liberate the amino group, form the thiol-

glyoxaline ring, hydrolyze and decarboxylate to obtain II.

Highly pigmented resins were obtained when the condensation was carried out.

e. Through a series of reactions, the following compound was obtained:



X

Methylation of X led to liberation of $N(CH_3)_3$ and production of an unsaturated acid. In another variation using X, the amino group was replaced by bromine by the action of nitrosyl bromide. This compound was to be hydrolyzed and the ring then formed, to produce II. However, on hydrolysis, HBr was given off, followed by ring closure.

Harington and Overhoff believe that the only hope of synthesis rests on the action of trimethylamine on II, and that this acid will have to be obtained from an appropriate thiolglyoxaline; that is to say, the formation of the thiolglyoxaline ring cannot be postponed until the last stage.

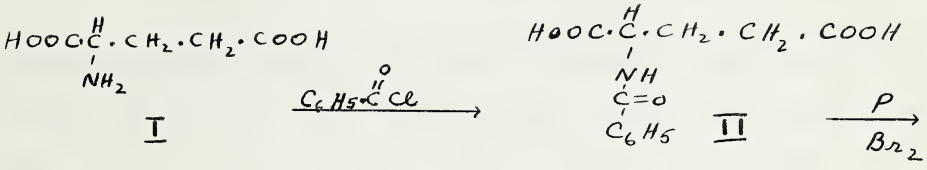
Dey (7) in 1937 described a new method of preparation of dl-thiolhistidine in hopes that it might lead to the ultimate synthesis of ergothioneine. In 1949, Winegard was reported to have been working on this problem (24).

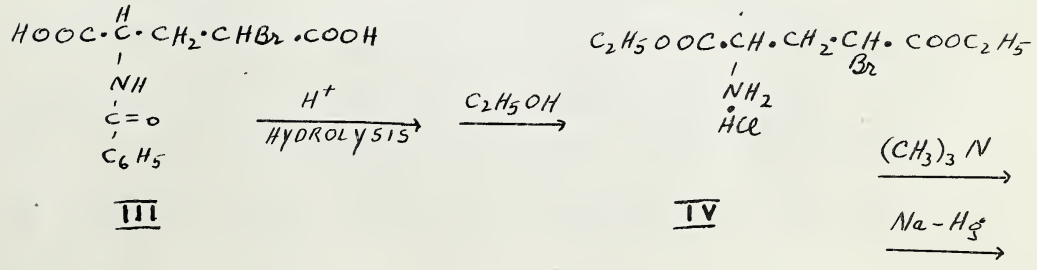
Harington and Overhoff used variations of the method of condensing an amino ketone with a thiocyanate salt to form the thiolglyoxaline ring. No work on ergothioneine

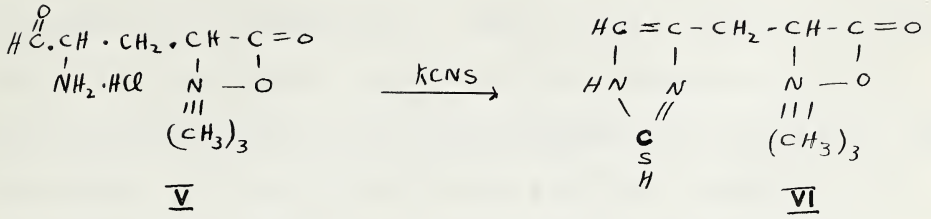
synthesis has been reported using the method described by Akabori (1) to form the thiolglyoxaline ring, i.e., condensing an amino aldehyde with a thiocyanate, so this method was incorporated into a series of reactions which, theoretically, could produce ergothioneine. These are described below in the experimental part. The ease with which the sulfur group and the trimethylamine group are removed in ergothioneine reduces the number of types of reactions that may be employed.

EXPERIMENTAL

1. Theoretically, it is possible to prepare ergothioneine using Akabori's method of ring synthesis starting with glutamic acid.







V VI

The object of this method was to obtain α-bromoglutamic acid diethyl ester (IV) which, after the bromine group had been substituted for by trimethylamine, was to be reduced by sodium amalgam to the aminoaldehyde (V). Then on condensation of V with a thiocyanate, ergothioneine (VI) should result. α-bromoglutamic acid has not been described in the literature and so its synthesis was attempted using, first, red phosphorus and bromine. The amine group was protected from intermediate acid chloride that would be formed during the reaction, by benzoylation.

Benzoylglutamic acid - This was prepared according to the method of Fischer (12) from glutamic acid, sodium bicarbonate, and benzoyl chloride. M.P. 138°C, yield 86% theoretical.

Bromination of benzoylglutamic acid

The method used was essentially that described by Eck and Marvel (10) for the bromination of ε-aminocaproic acid. Bromination was carried out in a three-necked flask fitted with a reflux condenser and gas trap, mechanical stirrer, and dropping funnel. Five gm. benzoylglutamic acid and

0.4 gm. red phosphorus were mixed and cooled in the flask by means of an ice-mixture. About 10 ml. bromine was used in all. It was added slowly at first with stirring until the vigorus reaction with the phosphorus had subsided, then added more quickly. The mixture was then refluxed at 60-70°C for 6 hours and then poured while warm into 30 ml. distilled water. A black, tarry material resulted with the evolution of heat. This was allowed to stand for an hour and then filtered. This material was dissolved in hot ethanol but could not be decolorized with charcoal. It was precipitated from cold ethanol by addition of water, whereupon about one-half the original amount of the tarry material came down again. The filtrate was concentrated in vacuo and allowed to stand. The solid that separated out gave a M.P. of 122°C on the second recrystallization, and a mixed melting point with benzoic acid of 122°C.

It appeared that the benzoylglutamic acid was hydrolyzed under these conditions of bromination. Probably the liberated amine group was then attacked by the intermediate acid chloride to form a resinous material. Attempts at bromination using varying temperatures and times of refluxing were likewise unsuccessful.

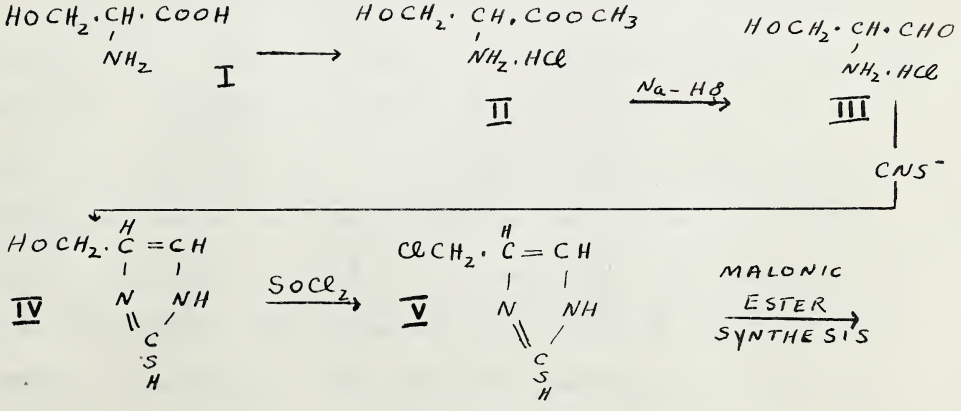
Another method of bromination was tried, that of Schwenk and Papa (30) for the bromination of dibasic acids. The conditions of the reaction are less drastic and it was hoped to affect a bromination of the benzoylglutamic acid without

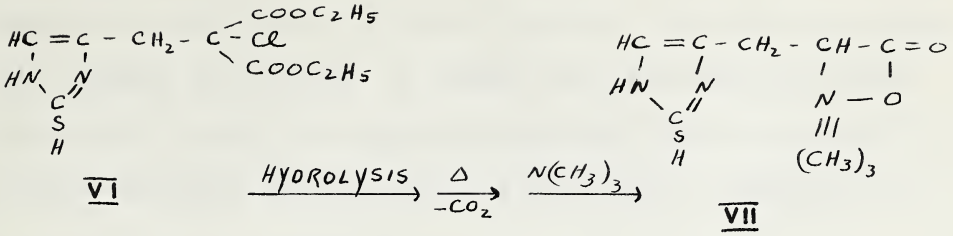
removal of the benzoyl group.

Five gm. benzoylglutamic acid, 13 ml. thionyl chloride, and 10 ml. bromine were refluxed for 2 hours in a three-necked flask equipped for bromination. The material reacted vigorously in the cold at first, and after a few minutes turned dark in color, with a solid separating out. When the refluxed mixture was poured into water, a tarry material resulted. As with the other procedure, benzoic acid was recovered from the reaction mixture.

It appears that the benzoyl group is easily removed from the amine group. The adjacent carbonyl group probably affects its reactivity, as Saunders (29), in preparing derivatives of monoaminodicarboxylic acids, found that the amine groups are much less reactive than those in other types of compounds.

2. Another method of synthesis was tried according to the following scheme:





VI

HYDROLYSIS, Δ, $-CO_2$, $N(CH_3)_3$

VII

The amino acid serine (I) was to be esterified and reduced by sodium amalgam[1] to its corresponding aminoaldehyde (III). On condensation with a thiocyanate, 2-thiol-4(or 5)-hydroxymethylglyoxaline (IV) should result. The synthesis of this compound was attempted by Harington and Overhoff but by other routes, as described in b and c of the introduction. IV was to be converted to the chloro derivative (V) by thionyl chloride or phosphorus pentachloride, and then by means of the malonic ester synthesis, α-halogeno-β-2-thiolglyoxaline-4(or 5)-propionic acid is prepared. Treatment with trimethylamine should give ergothioneine.

Serine methyl ester hydrochloride

The preparation was essentially that of Fischer and Jacobs (13). Ten gm. serine was covered with 300 ml. dry methanol (prepared according to Fieser (11)), and saturated with dry HCl gas. The clear liquid resulting was concentrated under reduced pressure (not above 50°C) to a thick syrup and allowed to crystallize. To complete the esterification, the operation was repeated with half the amount of methanol. The crystals, which formed in the

cold, were mixed with a little ethanol, filtered and washed with ethanol and ether. A second crop was obtained from the mother liquor by concentrating down again after addition of more methanol and HCl gas. Yield, 86% of theoretical.

Preparation of sodium amalgam.

Sodium amalgam was prepared according to the method in Lassar-Cohn (21). 2.5 gm. clean metallic sodium was submerged in 97.5 gm. mercury in a small beaker. The sodium was held in place by a pair of tongs. The operation was carried out in a fume chamber on account of the poisonous nature of the mercury fumes evolved. The reaction is violent and after about a minute the mixture bursts into flames. While still hot the sodium amalgam is stirred with a spatula until it solidifies. The amalgam is then obtained in a crystalline form. If allowed to stand while cooling it sets to a hard mass which must be pulverized.

Preparation of 2-thiol-4(or 5)-hydroxymethylglyoxaline

3 gm. serine methyl ester hydrochloride was dissolved in 14 ml. methanol and 30 ml. water in a 300 ml. beaker and kept at -10° C in an ice-salt mixture, with constant stirring. 80 gm. 2.5% sodium amalgam was added in small amounts over a period of 45 minutes. 5 N HCl was admitted dropwise during this time to keep the solution slightly acid. Stirring was continued for one-half hour longer

and the mercury then filtered off.

Benedict's test was positive at this point indicating successful aldehyde formation. The solution was concentrated under reduced pressure to 40 ml. and filtered from the insolubles. The solution turned dark brown during this step but Benedict's test was still positive. Then 3 gm. ammonium thiocyanate in 4 ml. water was added to the solution of the aldehyde with stirring and the mixture evaporated to dryness over a steam bath. After an hour, the reaction mixture gave a positive Hunter test[19] indicating formation of the thiolimidazole ring. The residue was warmed with 35 ml. absolute alcohol and filtered after cooling. The alcohol was distilled off in vacuo and the residue treated with 20 ml. water and charcoal. The filtrate from this was concentrated to a thick syrup and allowed to crystallize. 2.5 gm. of crystals were collected and washed with a small amount of cold water. However they gave a negative Hunter test and a positive Nessler's. The mother liquor still gave the positive Hunter test.

During attempts at isolation of this compound, material was being formed that could not be redissolved in hot alcohol. The mercury precipitation as described by Akabori was used in an attempt to further purify this thiolimidazole. The precipitate was decomposed by hydrogen sulfide and the filtrates from this step evaporated down. Crystals were obtained which gave a positive Nessler's, negative Hunter's test, and were insoluble in alcohol. It appeared that the

$$\xrightarrow{\text{HYDROLYSIS}} \xrightarrow[-CO_2]{\Delta} \quad HOOC \cdot \underset{\underset{NH_2 \cdot HCl}{|}}{CH} \cdot CH_2 \cdot CHCl \cdot COOH$$

III

The object of this method was to condense the chloro derivative of serine (II) with ethyl sodiochloromalonate first, and to form the thiolimidazole ring later. The α-bromoglutamic acid (III) thus built up would be treated as in method 1.

Serine methyl ester hydrochloride

This was prepared as for method 2.

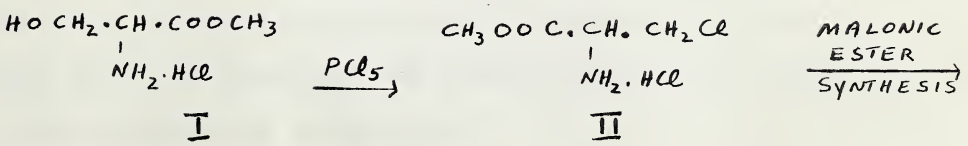
dl-α-amino-β-chloropropionic acid hydrochloride

This was prepared by the method of Fischer (14). 10 gm. serine methyl ester hydrochloride was dried over P_2O_5, then finely pulverized and suspended in 97 ml. freshly distilled acetyl chloride in a stoppered bottle. It was cooled by ice water and 14.6 gm. pulverized PCl_5 was added with shaking, in 3 portions in the course of 10 - 15 minutes. The original solid disappeared. The mixture was then shaken on the machine for one hour, and allowed to stand in the cold overnight. The flocculent precipitate which settled was washed with acetyl chloride and petroleum ether. Yield 41%; M.P. 125° - 130°C with browning and evolution of gas.

compound was unstable and was decomposing since any ammonium salt impurities should not be carried through the mercury precipitation. During further trials at synthesis of this compound, potassium thiocyanate was used instead of the ammonium salt, for the formation of the ring. After the mercury precipitation, the filtrate was concentrated to about 4 ml. Material crystallized out which gave a positive Nessler's and an ammonium test with sodium hydroxide and litmus. Since none of the reactant materials contained the ammonium ion, the thiolimidazole ring was assumed to be decomposing. This might explain why Harington was unable to hydrolyze compound VIII successfully, as mentioned in the introduction. This compound bears a resemblance to its analogous member in the glyoxaline series, hydroxymethylglyoxaline, which Pyman (28) found to decompose when distilled under 20 mm. pressure.

The above method of synthesis was discontinued and a variation of it attempted, but this was without success also. This was based on the following series of reactions.

2.

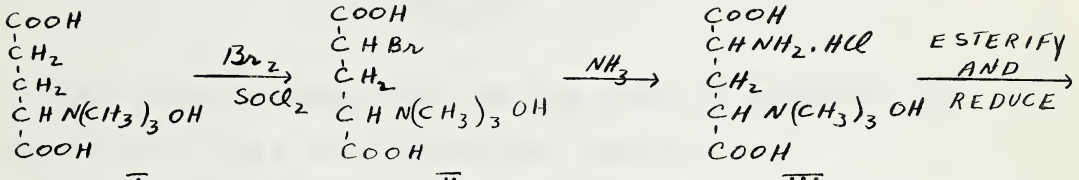


Fischer states that the crystallization of the dl-compound is poor and the purification is difficult. Previous trials at making this compound gave very poor yields, but the preparations exhibited the characteristic melting point with browning and evolution of gas. No success was had at crystallizing it from a methanol solution by ether, so the condensation with ethyl sodiomalonate was to be attempted without further purification of the product. If the condensation were successful, then the chloromalonate would be used. The HCl group of the chloroserine ester must be neutralized before condensation with the sodiomalonate, otherwise it would react before the chloro group. It was decided to neutralize the group, then add the free ester to the malonate for condensation. A 2% solution of sodium in dry methanol was used, with phenolphthalein as indicator. Sodium chloride precipitated out on addition of the sodium and the solution turned orange. Because of this, an attempt was made to recover the original material to make certain that the addition of sodium had not destroyed it. HCl gas was passed in to bring the solution to the acid side. It was concentrated to dryness in vacuo and taken up in dry methanol. On precipitation with ether, no chloro-compound was regenerated.

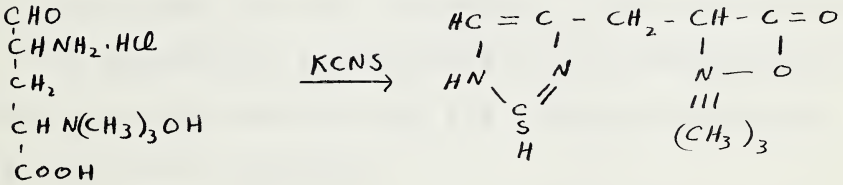
It was later learned that free α-amino esters on heating tend to form the dimolecular cyclic amides, and that free serine methyl ester itself, loses alcohol spontaneously to

form the diketo-piperazine (2). So this method was discarded since the chloroserine ester during condensation with the malonate would probably cyclize.

4. Another theoretically possible mechanism that was devised used trimethyl-d-glutarobetaine (I) as the starting material.



I II III



IV V

(I) was described by Dakin and West (6). It is soluble in water, insoluble in alcohol and ether and melts at 211° - 213° C. It was reported to be stable and on warming with strong alkali does not split off trimethylamine. Conflicting reports (25) have been published on the compound obtained by methylating glutamic acid;however, its preparation according to Dakin and West, who report obtaining the compound formulated as above, was attempted but without success. No crystals were formed from the final syrupy aqueous concentrate, and precipitation of this concentrate through addition of alcohol gave a material of M.P. greater than 250°C,with blackening. The material contained no nitrogen on analysis. Because of

the difficulties encountered in obtaining any amount of starting material and because of the uncertainty in the literature regarding its correct formulation, it was deemed inadvisable to continue with this method.

SUMMARY

1. Four possible mechanisms for the chemical synthesis of ergothioneine have been devised and tested.
2. Bromination of benzoylglutamic acid lead to production of a tarry mass with the liberation of the benzoyl group.
3. The preparation of 2-thiol-4(or 5)-hydroxymethylglyoxaline has been described and its isolation attempted. It was found to be unstable.
4. Method 3 was discarded because of the possibility of the free amino acid ester losing alcohol and forming the cyclic amide.
5. Trimethyl-d-glutarobetaine could not be prepared satisfactorily as a starting material for the fourth method. Therefore this method of synthesis was also discarded.

PART B - BIOCHEMICAL SYNTHESIS

INTRODUCTION

The presence of ergothioneine in the red blood cell has not been fully explained. Eagles and Vars (9) claim that diet, to a certain extent, affects the levels in animals. Potter and Franke (27) believe that ergothioneine is entirely exogenous in origin, stating that variations in pathological bloods are probably a result of the dietary regimes of the patients. The only diet found by Eagles and Vars, and Potter and Franke, to increase the ergothioneine level in blood was corn. The first named authors believe that an amino acid, 2-thiolhistidine, is probably present in corn and is the precursor of ergothioneine. However, no one has, as yet, demonstrated the presence of this amino acid. The latter authors give no suggestion as to the precursor of ergothioneine, but state that it is not likely to be glutathione, methionine, or thiolhistidine.

If there is a precursor of ergothioneine in certain diets, the mechanism by which it is transformed into ergothioneine and eventually incorporated into the red blood cell is quite obscure. If the ergothioneine is produced in the body during the normal course of metabolism, then a study of factors and pathological conditions affecting theblood level may throw some light on its presence and

role in the organism.

The investigation into the biochemical synthesis took the form of a study of the effect of certain dietary factors on the blood ergothioneine level, in an attempt to determine whether ergothioneine is exogenous or endogenous in origin. If it were exogenous in origin, the problem was to determine the precursor(s).

During the course of this work, some interesting leads concerning the mechanism whereby ergothioneine is incorporated into the cell have arisen, and these are reported below.

METHOD

Experimental Animals - Weanling and adult male albino rats, Wistar strain, were used for all experiments. They were housed in metal cages and given abundant food and water. Weight gains were recorded for the rats on special diets.

Collection of blood sample - One to 1.5 ml. tail blood was collected by ''milking'' the blood, with stirring, into a small porcelain crucible containing 2 mgm. potassium oxalate monohydrate. Either 0.25 or 0.5 ml. blood was taken for the ergothioneine determination, and the remainder was pipetted into a hematocrit tube of approximately 1 ml. volume and spun at 3000 r.p.m., for 30 minutes.

Determination of ergothioneine - Ergothioneine was determined in 0.5 and 1 ml. aliquots of the protein-free filtrates of the blood according to the method of Hunter (19).

Diets - The synthetic diets used contained the following:

McCollum salts	4%
cod liver oil	2%
crisco	3%
protein plus sucrose	91%

To one kilogram of the above diet were added 10 mgm. thiamine hydrochloride, 10 mgm. niacin, 10 mgm. pyridoxine hydrochloride, and 20 mgm. calcium pantothenate all dissolved in 10 ml. 70% ethanol; and 20 mgm. riboflavin distributed in

3 gm. sucrose. Choline chloride dissolved in 4 ml. 70% ethanol was added at a level of 0.1%.

The fat, protein, and carbohydrate levels were varied in the diets at the expense of each other.

EXPERIMENTAL

I - THE FATE OF INGESTED ERGOTHIONEINE.

The effect of ingestion of ergothioneine on blood ergothioneine levels.

The only suggestions in the literature as to precursors of ergothioneine have been 2-thiolhistidine (9) and histidine (18). Since ergothioneine occurs in most mammalian bloods, i.e., it can be ingested with meats, and since it is present in some plant foods, mushrooms, dandelions, and possibly corn and flax (26), the following experiment was designed in order to determine whether ergothioneine, existing as such in the diet, could affect the blood level.

Three adult rats (250 - 300 gm.) were force fed from a pipette, a total of 100 mgm. ergothioneine each, in doses of 10 mgm. every 24 hours. Blood was taken 5 times the first day, 2 times the second day, and once a day thereafter. Blood was taken from the control rats at the same times, except that the 6, 30, and 48 hour bleedings were omitted. During the experiment, the animals were fed a 1:1 mixture of Purina and Victor Fox chow, finely ground and mixed to ensure homogeneity of diet. The results are shown in Table 1.

Table 1 - The effect of ingestion of ergothioneine on the blood ergothioneine level.

Ergothioneine-fed rats					Control rats				
Time after feeding	Mgm. ergothioneine per 100 ml. corpuscles				Time after feeding	Mgm. ergothioneine per 100 ml. corpuscles			
	Rat I	II	III	Average		Rat IV	V	VI	Average
0 hours	17.0	16.8	16.6	16.8	0 hours	14.5	15.4	12.2	14.0
1½	14.8	15.4	16.5	15.5	1½	13.0	14.7	12.1	13.3
3	17.7	15.8	15.3	16.3	3	13.1	17.0	15.0	15.0
6	16.0	17.4	16.6	16.7					
11	15.0	14.9	14.9	14.9	11	14.9	14.7	16.0	16.5
24	18.0	16.4	15.8	16.7	24	15.3	16.2	15.9	16.0
30	17.6	16.5	17.2	17.1					
48	24.2	22.1	22.7	23.0					
72	21.4	20.2	22.7	21.4	72	18.2	20.0	22.0	20.1
5 days	25.0	25.9	25.0	25.3	5 days	14.5	16.2	17.2	15.9
*9	33.4	29.2	33.2	31.9	9	14.4	15.4	14.1	14.6
14	48.4	42.6	40.0	43.7	14	16.5	19.6	18.0	18.0
19	37.4	36.4	-**	36.9	19	13.9	16.0	14.9	14.9
23	32.6	32.0		32.3	23	16.0	18.5	17.1	17.2
29	31.2	25.8		28.5	29	11.0	15.1	16.2	14.1
44	19.8	17.9		18.9	44	13.1	14.2	12.9	13.4

* Ergothioneine feedings discontinued.

** Rat sacrificed for determination of ergothioneine in its organs.

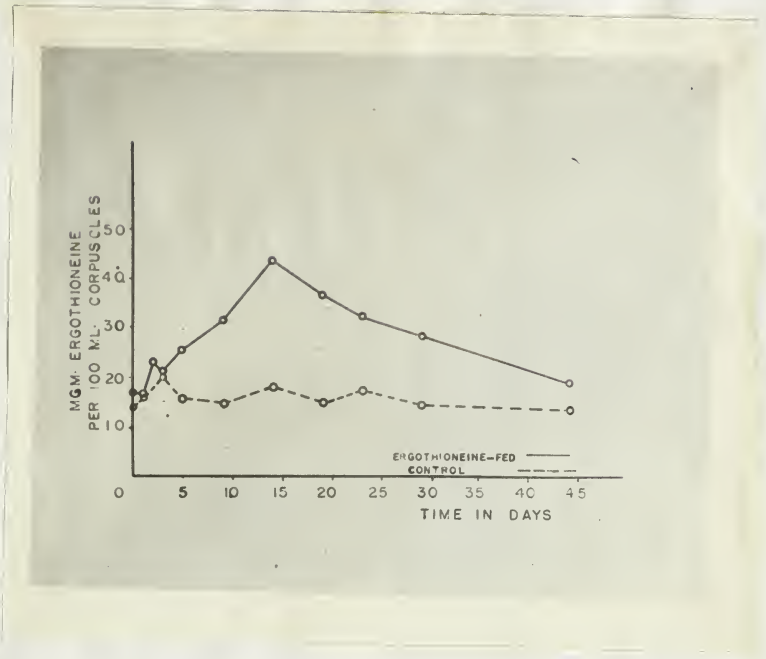


Fig. 1.- Graph of results from Table 1.

Fig. 1 shows that ingested ergothioneine causes increases in the blood level of this substance in rats about 4 or 5 days after the first feeding. The feedings were discontinued on the ninth day and it took from 3 to 5 weeks for the levels to approach normal values. No significant increase was detected in the first 30 hours after feeding.

Excretion of the ingested ergothioneine

Urine and feces were collected from the ergothioneine-fed rats over a period of 96 hours. Ergothioneine was determined on 10, 20, 30, 40, and 50 µl. aliquots of the 24, 24 - 48, and 48 - 96 hour urine specimens, by the method developed by Siluch (31) of this laboratory using the paper chromatography technique. Hot water extracts of the feces were tested in the same manner. Both urine and feces extracts over the 96 hour period were negative for ergothioneine as tested by this method. It was therefore concluded that ergothioneine was not excreted, as detectable ergothioneine, when this compound was fed orally.

Ergothioneine content of various organs

Since the ergothioneine eventually increased in the blood, and since it was not excreted (as ergothioneine), we thought that perhaps it could be broken down in the gastro-intestinal tract, its products absorbed, and the ergothioneine resynthesized from all or some of the products. This may have been the reason for its not being detected in the blood stream shortly after feeding. Or the possibility exists that the ergothioneine was stored in some organ of the body (especially the mucosa of the small intestine) and that it was slowly released, in undetectable amounts, to result in a gradual rise of concentration in the red blood cells.

Rat III was sacrificed on the fourteenth day and determinations of ergothioneine on extracts of its organs were carried out in an attempt to determine the location of ergothioneine stores, if any. For a control, the organs of a normal rat were also analyzed for ergothioneine. The method used for extraction was the same as that described below for liver ergothioneine determinations, except that the extraction volumes varied according to the size of the organ. The results are shown in Table 2.

Table 2 - Ergothioneine content of various organs of an ergothioneine-fed rat and a normal rat.

Rat	Blood (mgm.% corpuscles)	Liver	Lung	Kidney	Spleen	Marrow	Small Intestine
		(mgm.% wet weight)					
1	38.0	40.8	5.7	17.4	21.0	Int.	no ergothioneine detectable
2	10.8	14.8	Int.	Int.	Int.	Int.	-

Int. = interference colors given by the diazo test.
Rat 1 - ergothioneine-fed.
Rat 2 - normal.

No evidence has been found that ergothioneine was stored in the intestinal mucosa. The only organ that contained ergothioneine in significant amounts was found to be liver. From the values obtained for the livers in this experiment, 100 gm. liver contains more ergothioneine than 100 ml.

corpuscles, thus more than 100 gm. corpuscles. Since liver tissue contains a greater percentage of water than the red blood corpuscles, then on a dry weight basis, these livers are more concentrated in ergothioneine than the red blood cells. Therefore, further ergothioneine determinations were carried out on other livers to confirm these findings, and these are reported below. The presence of ergothioneine in the other organs tested could result from their content of blood in which ergothioneine was present in abnormally high concentrations. These same organs, other than liver, in normal animals gave poor ergothioneine tests, and mostly showed interference colors.

Ergothioneine in Liver Tissue

The following method of extracting and determining the ergothioneine content of liver was adopted:

The rat was killed by decapitation and the liver was excised and washed with distilled water. It was dried with a clean cloth, weighed and placed in a boiling tube in a boiling water bath for 15 minutes. The contents of the tube were then ground in a mortar to a homogeneous paste, transferred, with washings, to a 100 ml. volumetric flask and again placed in a boiling water bath for 20 minutes. The flask was cooled and made to volume. A one ml. aliquot was diluted to 5 ml. with distilled water, and the liver brei was centrifuged off. The supernatant was then

treated with 1 drop of Goulard's and 1 drop of 10% NaH_2PO_4 solution as in the blood determination. Diazo tests were done on the final filtrate. A few of the tests on the extracts showed interference, but the results of only those livers whose extracts gave the typical red-purple diazo color are reported. Table 3 shows some ergothioneine values on rat livers, with blood ergothioneine levels reported for comparison. Results are included in this table for diabetic rats because it was noted by Gee (16) that abnormally high levels of ergothioneine were present in the blood of such animals.

Table 3 - Ergothioneine content of some rat livers.

Rat	Weight fresh liver (gm.)	Ergothioneine mgm.% wet weight	Blood ergothioneine mgm.% corpuscles
Ergothioneine-fed	8.03	35.4	25.0
"	12.24	40.8	38.0
Diabetic	6.83	27.1	18.8
"	9.94	21.8	22.6
"	10.78	24.6	22.8
"	8.17	19.5	12.5
"	14.23	31.5	19.3
Normal	8.35	21.7	14.7
"	4.82	14.8	10.8

In all the livers analyzed, the ergothioneine content was found to be greater than in the corpuscles. Possibly the liver has some role in removing the ergothioneine from the red blood cell, or in storing the ergothioneine until it is incorporated into the cell.

Application of corrections to blood ergothioneine values

In the experiment on ergothioneine ingestion, the first rise in the blood ergothioneine of both groups of rats was exhibited during the first 100 hours.(see Fig. 2). Because of the fairly severe blood loss in both groups of rats during the first few days of the experiment, it was necessary to consider exsanguination as a possible cause of this increase. Hunter's (19) and Fraser's (15) findings that plasma contributes to the whole blood ergothioneine color density were applied to the study of the rise in ergothioneine due to exsanguination. When the hematocrit is low, the plasma contribution is consequently increased. Then, when the ergothioneine of blood with a low hematocrit is calculated on the basis of 100 ml. corpuscles, this increased plasma effect is also multiplied, giving apparently higher ergothioneine values. The effect of exsanguination was studied over a period of 48 - 72 hours only, to parallel the rise indicated in Fig. 2, and it was concluded that the increased ergothioneine level was apparently due to a lowered hematocrit

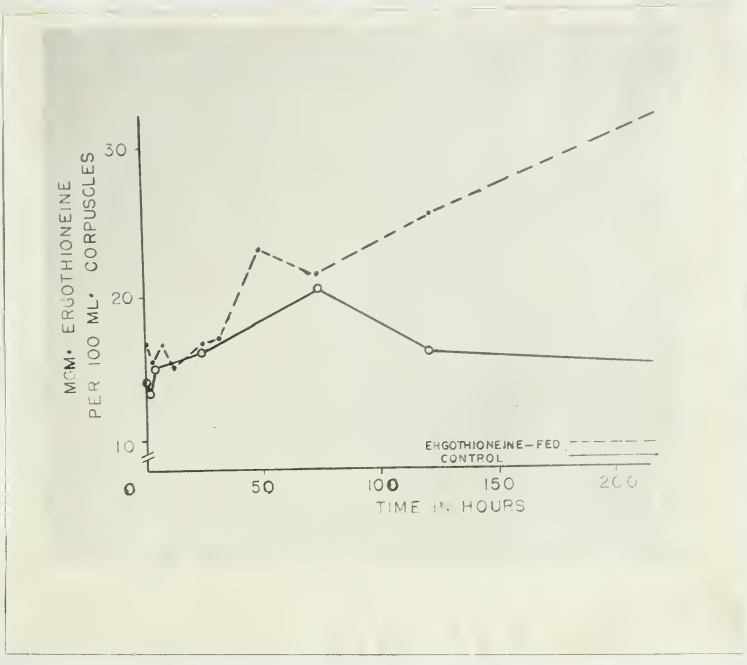


Fig. 2 - An enlarged section of Fig. 1.

and in some cases, a slightly increased plasma value. After the necessary corrections were applied to the ergothioneine values, any small increases in the blood ergothioneine then were considered insigmificant, and probably resulted from slight variations in the diet.

However, in later work (page 52) where exsanguination was followed over a period of 12½ days, it is clearly seen that exsanguination does cause an increase in blood ergothioneine. This increase does not become significant until after 72 hours, so this is why the first conclusions were erroneous.

The part of this study that had any value was the application of plasma corrections to blood ergothioneine values. By Fraser's method of freezing centrifuged blood, he was able to show that ergothioneine in the red cells plus the plasma effect in ergothioneine units equals the ergothioneine in the whole blood. Diazo tests on human plasma give a yellow color which represents in ergothioneine units, an average value of 1.0 mgm. ergothioneine per 100 ml. plasma. An average of 10 determinations on rat serum (serum was found to give the same value as plasma) gave a value in ergothioneine units of 1.5 ± 0.2 mgm. ergothioneine per 100 ml. serum. Fraser's freezing method applied to rat blood is not practical. The centrifuged cells, after freezing and thawing, cannot be pipetted because they are in the form of a very

thick paste. Therefore to confirm the belief that ergothioneine in the cells plus the plasma effect equals whole blood ergothioneine for rat blood as well as for human blood, the following data were collected.(see Table 4). Dilutions of the whole blood to produce varying hematocrit values on the same blood, were made with the serum rather than plasma because it was easier to obtain the serum free from hemolyzed cells. However, since both serum and plasma give the same ergothioneine color density, no error was introduced.

Table 4 shows that whole blood ergothioneine minus the plasma effect that is proportional to the amount of plasma in the blood, gives constant values for ergothioneine in the cells. The error introduced when ergothioneine values are not corrected for the plasma effect is significant in bloods of low hematocrit value, as seen in column 4(ergothioneine, mgm.% corpuscles).

Using the methods of calculations shown in the table, and the average value found for plasma (1,5), all ergothioneine values in the subsequent experiments were corrected for the plasma effect.

Table 4 - Plasma effect in whole blood ergothioneine

Rat	Hemato-ocrit	Ergothioneine mgm.% whole blood	Ergothioneine mgm.% corpuscles	Apparent Ergothioneine mgm.% plasma	Plasma effect	Ergothioneine mgm.% whole blood minus plasma effect	Ergothioneine mgm.% corpuscles (corrected)
1	38%	10.6	27.9	1.6	1.0(62% of 1.6)	10.6 - 1.0 = 9.6	25.3
	19	6.1	32.1	1.6	1.3(81% of 1.6)	6.1 - 1.3 = 4.8	25.2
2	39	11.6	29.8	2.0*	1.2(61% of 2.0)	11.6 - 1.2 = 10.4	26.7
	19.5	6.8	35.1	2.0	1.6(80.5 % Of 2.0)	6.8 - 1.6 = 5.2	26.6

* Higher value than normal due to slight hemolysis

II - THE EFFECT ON BLOOD ERGOTHIONEINE LEVELS OF DIETARY SUPPLEMENTS.

Effect of choline on blood ergothioneine levels.

Ashley and Harington (3) believe that 2-thiolhistidine can be methylated in vivo to produce ergothioneine. Therefore choline, acting as a source of transferable methyl groups, was studied in its effect on the blood ergothioneine level.

Five rats (approximately 160 gm. each) were fed the 10% casein diet with 1 gm. choline chloride per kgm. of diet added. Five control rats were fed the same diet, but without any choline. Blood was taken for ergothioneine determinations at 0, 1, 2, 3, 4, and 6 weeks. The results are reported in Table 5. The graph of the results (Fig. 3) shows that choline in this diet does not affect the ergothioneine level. In both the choline-plus diet and the choline-free diet, there is a slight decrease in the ergothioneine level until the third week, after which time the ergothioneine values drop rapidly and then remain more or less constant at a low level. This seems to be in agreement with the amount of time taken for the extra ergothioneine of the ergothioneine-fed rats to be removed from the blood.

The limiting factor in this experiment may be the casein itself. If some precursor is needed to be methylated to

ergothioneine, and this is not supplied by the diet, then choline, as a source of labile methyl groups, would not alone increase the blood ergothioneine.

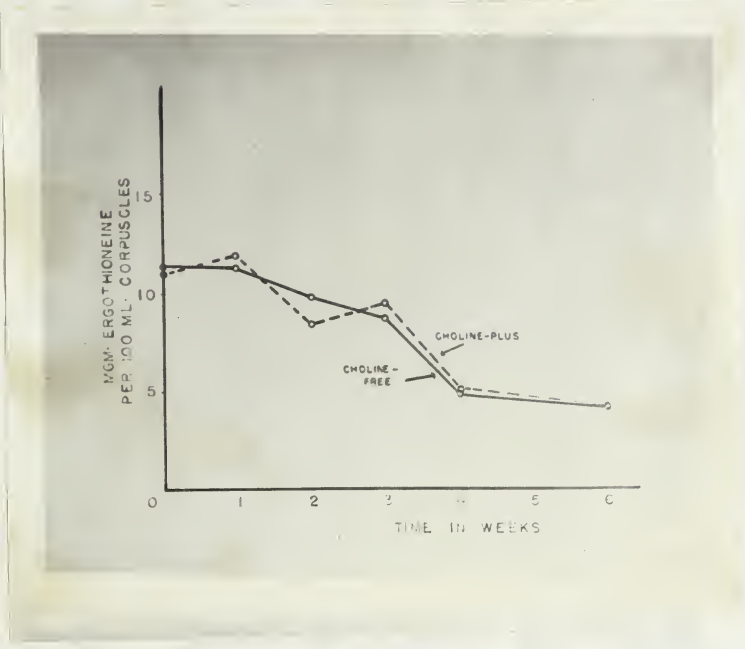


Fig. 3 - Graph of results from Table 5.

Table 5 - Effect of choline on blood ergothioneine.

Time in Weeks	Ergothioneine, mgm.% corpuscles					Average	Average weight	Ergothioneine, mgm.% corpuscles					Average	Average weight
	Rat I	II	III	IV	V			VI	VII	VIII	IX	X		
0	9.7	11.9	13.5	11.8	10.7	11.5	167	12.3	10.4	12.1	9.3	11.7	11.1	167
1	9.6	11.7	13.0	11.2	11.6	11.4	161	11.3	9.6	14.5	10.8	14.2	12.1	165
2	9.4	9.8	10.9	10.9	8.6	9.9	168	7.4	7.2	11.8	8.5	8.3	8.6	174
3	9.3	8.2	8.9	9.3	8.4	8.8	169	8.5	8.8	10.3	10.4	10.0	9.6	184
4	5.7	4.4	6.2	4.2	4.7	5.0	182	4.8	5.7	4.8	4.6	6.3	5.2	203
6	4.9	3.7	4.2	4.4	4.0	4.2	191	3.5	5.3	5.1	4.3	3.4	4.3	213

Rats I - V - on choline-free diet
Rats VI - X - on choline supplemented diet

Effect of histidine on blood ergothioneine

Because of the structural similarity between histidine and ergothioneine, it was thought that histidine might be a precursor of this compound.(18). Rats on corn diets show increases in blood ergothioneine (27) and in the albumin fraction of corn the histidine content is 4-5%, greater than in most proteins.

Histidine monohydrochloride was force fed from a pipette at a level of 60 mgm. per day, as a supplement to a low protein barley diet described by Tuba, Cantor, and Richards (36). This amount of histidine was calculated to be approximately twice the daily requirement on the basis that food consumption was 10 gm. per animal per day. The rats on the control diet were not fed the histidine supplement. The results are tabulated in Table 6. Rats on Purina Fox chow show increases in blood ergothioneine in less than four weeks, so this length of time was deemed sufficient for assaying other diets for their ability to increase blood ergothioneine.

Table 6 - Effect of low protein barley diet plus histidine on blood ergothioneine.

Time in Weeks	Ergothioneine mgm.% corpuscles (average of 4 rats)			
	Diet Plus histidine	Average Weight	Diet Control	Average Weight
0	8.9	153	8.1	154
2	6.8	151	5.1	154
4	5.4	172	4.1	180

Low protein barley diets result in a general decrease in blood ergothioneine, and histidine supplements have no effect in increasing the level. In case the overall deficiency of amino acids affected the possible conversion of histidine to ergothioneine, this experiment was repeated with a 10% casein diet supplemented with 60 mgm. histidine per day. The same decreases in ergothioneine were noted as with the choline diets, i.e., a slow decrease for about 3 weeks, and then a sharper drop (see Table 7).

Table 7 - Effect of 10% casein plus histidine on blood ergothioneine.

Time in Weeks	Ergothioneine mgm.% corpuscles (average of 4 rats)
0	9.5
3	6.9
4	3.9

From these experiments, it was concluded that histidine is not a precursor of ergothioneine.

Effect of methionine on blood ergothioneine.

Because of the presence of sulfur and labile methyl group in the methionine molecule, the effect of this compound in the diet on the blood ergothioneine was studied. Methionine was added to a 1:1 mixture of ground Purina and Victor Fox checkers at a level of 0.5%, and fed to a group of 5 adult rats (125 - 150 gm. each). The control group received the same diet but without added methionine. Table 8 shows that both groups exhibited a slight but insignificant rise in blood ergothioneine. The rats used for this experiment were taken from the stock cage where they had received mostly Victor checkers as food. The slight rise in ergothioneine noted in this experiment may have been a result of changing from this stock diet to the 1:1 mixture, described above.

Table 8 - The effect of methionine on blood ergothionine.

Time in Weeks	Ergothioneine mgm.% corpuscles (average of 5 rats)	
	Methionine diet	Control diet
0	12.6	11.2
2	10.3	9.9
4	13.9	11.4
6	15.5	14.6

Comparison of high fat, high protein, and high carbohydrate diets in their effect on blood ergothioneine.

Diabetic rats were observed to have abnormally high ergothioneine values (16). Since fat and carbohydrate metabolism is altered in this condition, the effect of diets with varying amounts of these substances on the blood ergothioneine level was studied.

Groups of 8 weanling rats were started on the following diets:

High fat	casein	10%	fat	40%	sucrose	46%
High protein	''	91	''	5	''	0
High carbohydrate	''	10	''	5	''	81
Control	''	40	''	5	''	51

An equal number of drops of blood from the rats on each diet was pooled in order to obtain enough for a determination. Blood was taken at 0, 2, 4, and 6 weeks. Average weights were recorded at these same intervals, and the results are reported in Table 9.

Table 9 - Comparison of the effect of high fat, high protein, and high carbohydrate diets on the blood ergothioneine.

Time in Weeks	High fat		High carbo-hydrate		High protein		Control	
	Ergothio-neine mgm.% corpuscles (E)	Aver-age Weight (W)	(E)	(W)	(E)	(W)	(E)	(W)
0	16.9	42gm.	18.2	40	15.3	38	19.4	45
2	9.3	55	8.4	56	4.6	60	5.0	80
4	5.3	57	4.3	67	1.5	75	1.9	116
6	4.5	64	3.0	73	1.4	87	1.3	154

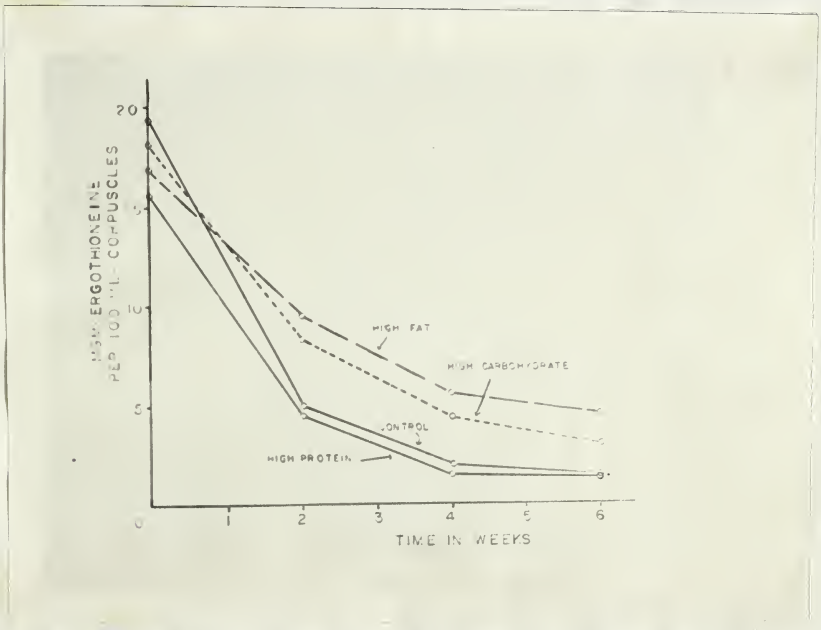


Fig. 4 - Graph of results from Table 9.

All types of diets cause a lowering of the blood ergothioneine. At the end of six weeks there was no ergothioneine detectable in the blood filtrates of rats on the control and high protein diets. Fig. 4 shows that the richer the diet is in casein, the more effective it is in lowering the blood ergothioneine. The reason for this is unknown. The control rats were placed on Purina Fox checkers at the end of the experiment. A month later these eight rats had an average blood ergothioneine value of 9.7 mgm.% corpuscles. It appeared quite probable that the casein lacked some factor necessary to increase blood ergothioneine, and that this factor was present in Purina Fox checkers. The fact that diets with increased content of casein show increased effectiveness in lowering blood ergothioneine levels may suggest also that either increased protein metabolism causes the ergothioneine to decrease or that there is some inhibitory effect on the ergothioneine production, in casein.

No noticeable symptons were observed in these rats other than poor growth on all but the control diet.

Effect of dried blood and dried liver supplements on blood ergothioneine.

Since our rats showed marked elevations in blood ergothioneine when they were taken off the synthetic diets and placed on Purina Fox checkers, we tried to determine the dietary factors responsible. Among the ingredients of Purina Fox chow that might cause a rise in blood ergothioneine were: animal liver meal, dried yeast, fish meal, meat meal, and soybean oil meal. It is possible for some of these ingredients to contain ergothioneine or some precursor.

We then determined the effect of dried blood and dried liver supplements on the blood ergothioneine. Two groups of seven rats each were fed diets of 10% casein plus 10% dried hog liver, and 10% casein plus 10% dried hog red blood cells. Ergothioneine determinations showed a level of 6.1 mgm.% corpuscles in the hog blood, but no value was obtained for the hog liver because of interference. The average results for the seven rats on the two diets are reported in Table 10.

Table 10 - The effect of dried liver and dried blood supplements on blood ergothioneine.

Time in Weeks	Dried blood		Dried liver	
	Ergothioneine mgm.% corpuscles (average of 7 rats)	Average Weight	Ergothioneine mgm.% corpuscles (average of 7 rats)	
0	10.9	120	157	7.8
2	10.1	159	211	6.5
4	12.8	203	249	5.6
6	13.8	240	298	5.3

Rats receiving the dried liver supplement showed a decrease in blood ergothioneine, while those receiving the blood supplement showed a slight increase. This increase was not marked but it did show that ergothioneine was being replaced, to keep the level from falling.

About the time this experiment ended, we had at our disposal some rats that had been fed on 40% casein, and consequently had low ergothioneine levels. These rats were given the dried blood supplement to determine how much of an increase in ergothioneine would result by starting with an initial low level. The experiment was concluded at the end of four weeks. Table 11 shows the results.

Table 11 - Rise in ergothioneine levels of rats fed dried blood supplements after a 40% casein diet.

Time in Weeks	Ergothioneine mgm.% corpuscles				
	Rat I	II	III	IV	Average
0	4.4	4.2	5.0	5.0	4.6
2	5.7	5.0	6.0	6.2	5.7
4	11.2	11.3	12.3	12.9	11.9

Table 11 shows that 10% casein in the diet does not inhibit the ergothioneine rise to any extent (Fig. 4, page 38, suggests the possibility that there is some inhibitory effect shown by casein which could prevent the ergothioneine level from rising). The above experiment also indicated the importance of using rats with initially low ergothioneine levels when assaying diets for ergothioneine-producing ability. The reason for this will be discussed following a later experiment.

The factor in blood supplement responsible for the rise in ergothioneine could be ergothioneine itself. Further experiments should be carried out with blood supplement that has had its ergothioneine destroyed, perhaps through mild oxidation, to ascertain whether any compound other than ergothioneine could cause this rise.

On the factor in Purina Fox chow responsible for its ergothioneine-producing ability.

There was no ergothioneine detectable in Purina Fox checkers; however, a water extract, when analyzed, did give a yellow coupling color with a slight orange coloration on addition of base.

Water and ether extracts were made of finely ground Purina checkers and these along with the residue were fed to groups of three rats each which had previously been on a 40% casein diet.

The diets were made up as follows:

1. Control (finely ground Purina checkers).
2. H_2O extract from 1 kgm. checkers was added to 1 kgm. ground checkers. 300 gm. checkers were extracted 3 times with a total of 2500 ml. hot water, acidified with several drops of acetic acid. This extract was concentrated in vacuo to a thick paste and mixed with 300 gm. dry, ground checkers.
3. The fat extract from 1 kgm. checkers was mixed with 1 kgm. ground Purina checkers. One kgm. of the residue from the water extract was ether extracted for 16 hours. The ether extracts were concentrated and added to 1 kgm. dry, ground checkers.
4. Residue from the ether extract.

The results are shown in Table 12.

Table 12 - The effect of Purina checker extracts on the blood ergothioneine level.

Time in Days	Ergothioneine mgm.% corpuscles (average for 3 rats)			
	Residue	Control	Water Extract	Ether Extract
0	4.2	4.6	4.4	4.4
6	4.7	4.5	4.7	4.5
10			5.1	
18	4.3	8.2	10.1	8.6
28	3.5	10.0	10.5	9.5

Table 12 shows that the residue does not contain the ergothioneine precursor. The results from the other diets are not significant enough to draw any conclusions. At the time this experiment was started, results from experiments described on pages 37 and 41 were known, i.e., that casein diets caused a lowering in the blood ergothioneine and that rises in ergothioneine in rats fed casein plus blood supplement were only slight (perhaps because of an inhibitory effect by the casein), so the extracts for this experiment were added to the control diet rather than to a diet with casein as base. It was hoped that if the ergothioneine-producing factor were contained in the water or ether extract, a much greater rise would be given with the control

plus water extract, or the control plus ether extract, than with the control diet alone (it was known from the experiment on page 20, that feeding ergothioneine can raise the blood level to a high value). Concurrently with this experiment was run another reported on page 42, but its results were not known in time to apply to this experiment. It appears that ergothioneine-producing diets do not cause much of an increase in blood ergothioneine levels when this substance is already present in fairly high concentrations, but if the rats begin with an initially low level, the rise is quite significant. Also from that experiment, it was learned that 10% casein as a basal diet, did not exert any appreciable inhibitory effect on the rise in blood ergothioneine.

The water or ether extract may have the ergothioneine-producing factor present, but any rise is difficult to interpret when all three diets produced a more or less maximum level within a month. Therefore this experiment should be repeated, adding the extracts to diets with 10% casein as base.

III - THE FATE OF INJECTED ERGOTHIONEINE.

The removal of injected ergothioneine from the blood.

In a trial experiment, one adult rat was injected intravenously with 10 mgm. ergothioneine in normal saline, through the tail vein. Tail blood was taken 1 hour after injection and the rat was killed by decapitation and the blood was collected for serum. The liver was analyzed. No urine could be aspirated from the bladder for the estimation of ergothioneine. The results are tabulated below, in Table 13.

Table 13 - Ergothioneine in blood after injection of 10 mgm., intravenously.

Time	Before Injection	One Hour After Injection
Hematocrit	59.5%	48%
Ergothioneine mgm.% whole blood	4.4	4.4
Ergothioneine mgm.% corpuscles	7.4	8.3
Apparent ergothioneine mgm.% plasma	1.5	2.6*
Plasma effect	0.6	1.3
Ergothioneine mgm.% whole blood minus plasma effect	3.8	3.1
Ergothioneine mgm.% corpuscles(corrected)	6.4	6.5
Liver - no ergothioneine was detectable, although an intense yellow color was produced in the coupling reaction.		

* This increase in the plasma value could not be attributed to ergothioneine, as no typical ergothioneine color was produced.

From the above table, it can be seen that all the ergothioneine has been removed from the plasma within an hour. There was no significant rise in the ergothioneine in the corpuscles, so it unlikely that ergothioneine can diffuse from the plasma into the red cells.

Nephrectomized rats injected with ergothioneine.

From the preceeding experiment, we concluded that either some organ was removing the ergothioneine from the blood stream or it was being excreted by the kidneys. If the latter were the case, there is a possibility that the ergothioneine could be excreted in some undetectable form. Bilateral nephrectomies on rats would eliminate altogether this possible route of excretion and more positive information would result than attempting to determine ergothioneine in the urine.

Bilateral nephrectomies were performed on two rats followed by injections of 10 mgm. ergothioneine in normal saline into the tail vein. The results from rat I are tabulated in Table 14.

Table 14 - Blood ergothioneine level in a nephrectomized rat injected with 10 mgm. ergothioneine.

Rat I	Ergothioneine mgm.%				
Time after Nephrectomy	Whole Blood	Hematocrit	Corpuscles	Plasma	Corpuscles (corrected)
Before Nephrectomy	9.7	58%	16.7	1.5	15.6
2½ hours	10.2	57.5	17.8	-	-
*2¾ "					
3¾ "	13.5	50.0	27.0	-	-
4¾ "	10.4	40	26.0	6.4	16.5

* Rat given an intramuscular injection of 10 mgm. ergothioneine.

Table 14 shows that the greater part of the injected ergothioneine was removed from the plasma but not by the kidneys. Whereas all the ergothioneine was removed from the plasma in an hour in normal rats, there still remained about 0.7 mgm. of the injected ergothioneine circulating in the blood of the nephrectomized rat two hours after the injection. This value (0.7 mgm.) was obtained from the following approximations:

Approximate volume of blood in the rat (10% of body weight - 250 gm.)	25 ml.
Hematoctrit	40%
Approximate volume of plasma	15 ml.
Normal plasma value in ergothioneine units, mgm.% plasma	1.5
Two hours after injection, plasma value, mgm.% plasma	6.4
Ergothioneine in the plasma due to the injection=(6.4 - 1.5) mgm.% plasma	4.9
Ergothioneine in 15 ml. plasma	0.7 mgm.

The fact that this amount of ergothioneine remained in the plasma of the nephrectomized rat may mean that the ergothioneine is excreted by the kidney after a certain saturation is reached by the organ or agents responsible for removing the greater part of the ergothioneine from the plasma. With the kidneys removed, this ergothioneine then remains in the plasma. We thought that if another injection of ergothioneine were given, the ergothioneine in the plasma should rise considerably. This was attempted with rat II. The results are given in Table 15.

Table 15 - Plasma ergothioneine in a nephrectomized rat following two injections of ergothioneine.

Rat II	Ergothioneine mgm.%				
Time after Nephrectomy	Whole Blood	Hematocrit	Corpuscles	Plasma	Corpuscles (corrected)
Before Nephrectomy	7.1	50%	14.2	0.8	13.4
*0 hours					
1 ''	8.7	42	20.6	4.1	15.0
**2 ''					
26 ''	11.8	38.5	33.8	8.9	16.3

* Rat was given an intravenous injection of 10 mgm. ergothioneine.

** Rat was given an intramuscular injection of 10 mgm. ergothioneine.

Most of the ergothioneine had been removed from the plasma of rat II within an hour. The second injection of 10 mgm. ergothioneine did not produce a marked rise in the plasma. About 1.2 mgm. of the total 20 mgm. injected, remained circulating in the plasma 26 hours after the first injection, using the approximations shown above. This second experiment showed that the organ or agents reponsible for removing the ergothioneine from the plasma was still able to remove the greater portion of the ergothioneine introduced with the second injection.

The effect of injected ergothioneine on the blood ergothioneine level.

In view of the facts that most of the injected ergothioneine was removed from the plasma of the nephrectomized rats (i.e., it was not all excreted), and that it took 4 or 5 days for the ergothioneine to increase in the blood after feeding it to normal rats, the effect of the injected ergothioneine was followed over a short period of time in normal rats. At the conclusion of one of the experiments performed by other workers in this laboratory, six rats were available that had been injected intravenously with 5 mgm. ergothioneine. Rats 4, 6, 8, and 11 had received alloxan with their injection. Table 16 shows that after several days the ergothioneine rises in the red blood cells, since no ergothioneine was demonstrated in the plasma. No controls were used for this trial experiment because from previous work, rats on the stock diet were known to have an insignificant rise in their ergothioneine levels over this short period of time.

Table 16 - The effect of injected ergothioneine on the blood ergothioneine level.

Time after injection	Ergothioneine mgm.% corpuscles Rat 1	2	4	6	8	11
0 hours	8.8	10.0	9.8	11.5	11.1	11.9
18 "	9.4	11.3	10.4	11.0	13.2	11.4
5 days	19.5	20.5				
6 "			22.6	25.5	20.8	23.7

Applying this information to the feeding experiment with ergothioneine, we can easily see how it is possible for the ergothioneine to be absorbed through the wall of the intestine into the blood stream. Here it is quickly removed by some organ, and therefore cannot be detected in the blood by the diazo test. Then four or five days later, it appears in the red blood cells. The problem of how this injected ergothioneine was removed from the blood stream was not further investigated.

The effect of exsanguination on blood ergothioneine levels.

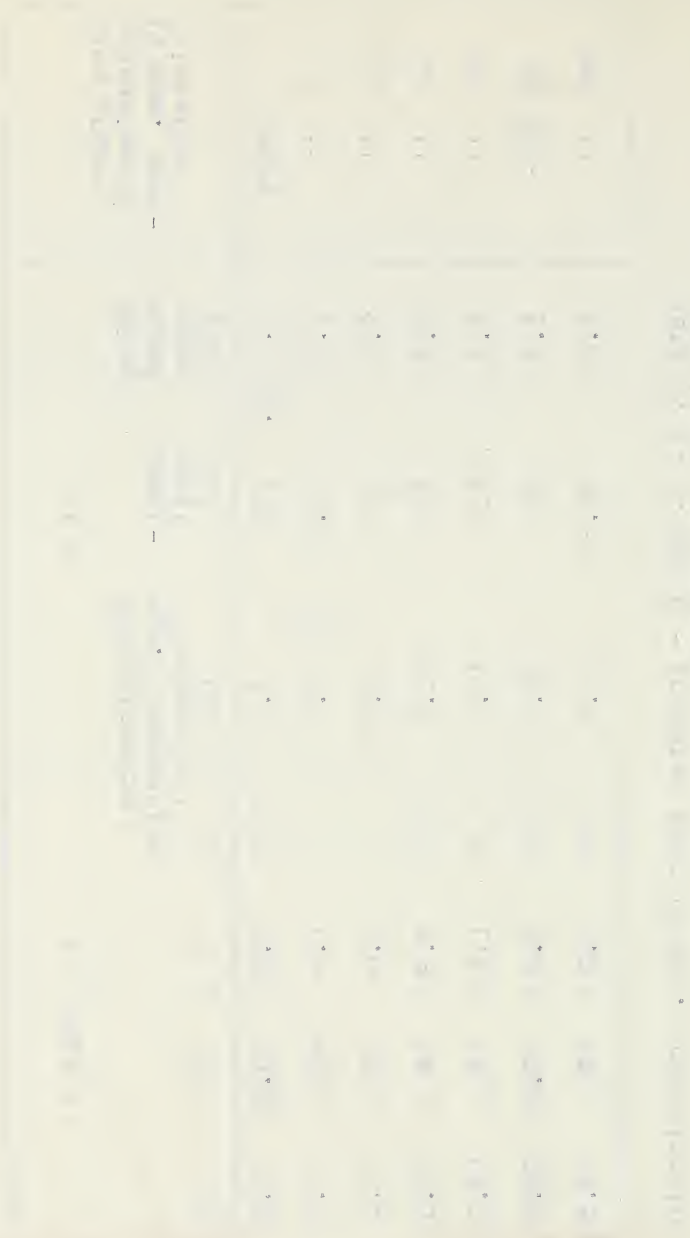
Ergothioneine does not appear to diffuse from the plasma into the red blood cell in vivo (see experiment, page 46), nor does it seem probable that after 4 or 5 days, the injected ergothioneine is taken up, all at once, by the circulating old red blood cells. However, this lag in the appearance of ergothioneine in the red cell may mean that the ergothioneine is mobilized for incorporation into new cells, and not until these new cells,with their increased ergothioneine content, are thrown into circulation will the blood ergothioneine level increase. A trial experiment was devised to obtain some information regarding this. Two rats (#2 and #5) were given one injection of 10 mgm. ergothioneine and were made anemic through continuous bleeding. If the appearance in the blood stream of the injected ergothioneine awaits production of new cells, then the rats (#2 and#5) which have been exsanguinated

(reticulocyte production probably stimulated), should show a more rapid rise in blood ergothioneine than rats (#4 and #6) which have been injected but bled less often (normal reticulocyte production). Two control rats (#1 and #3) were given no injections but were bled at the same times as rats 2 and 5. Plasma ergothioneine determinations were done for all blood samples. The results are reported as mgm.% corpuscles, corrected for the plasma effect, in Table 17.

Table 17 - The effect of exsanguination on blood ergothioneine.

Time after injection of 10 mgm. ergothioneine	Rat 2			Rat 5			Rat 4			Rat 6			Rat 1			Rat 3		
	Volume blood taken (V)	Hematocrit (H)	Ergothioneine mgm.% corpuscles (corrected) (E)	V	H	E	V	H	E	V	H	E	V	H	E	V	H	E
0 hours	2.3 ml.	47%	7.8	1.8	50.5	5.5	1.1	46	5.4	1.0	51	6.1	1.8	50	7.8	1.8	52.5	16.5
1 "	1.5	44.5	7.7	1.5	45	5.8	-	-	-	-	-	-	1.5	-	-	1.5	-	-
18 "	2.0	39	8.6	2.0	38	8.1	-	-	-	-	-	-	2.0	-	-	2.0	-	-
48 "	2.3	36	11.1	2.2	34	10.8	-	-	-	-	-	-	2.2	40	10.0	2.5	41	19.8
72 "	1.5	32	15.6	1.5	31	14.0	-	-	-	-	-	-	1.5	35	10.3	1.5	36	19.2
8½ days	1.5	40	18.9	1.5	40.5	20.8	1.0	50.5	11.4	1.0	53	11.9	1.5	45	12.6	1.5	41.5	25.5
12½ "	1.0	50.5	21.9	1.0	46	24.3	1.0	51.5	14.9	1.0	48	15.5	1.0	51	15.7	1.0	51.5	26.8

Rats 2, 5, 4, and 6 - injected with 10 mgm. ergothioneine.
Rats 1 and 3 - controls



All four rats that were injected show increased ergothioneine levels, but the two rats that have been bled frequently (#2 and #5) show the greatest increase. Ergothioneine has also increased in the blood of the control rats that were exsanguinated. From previous experiments, we knew that rats on stock diets that are not bled often, show fluctuations in the ergothioneine level of only 1 or 2 mgm.% corpuscles in this same short period of time. Therefore, two other control rats not injected with ergothioneine and bled at the same times as rats 4 and 6 were not considered necessary.

From this trial experiment it is quite evident that exsanguination causes an increase in the blood ergothioneine. If we assume in this experiment that reticulocyte formation was increased because of the exsanguination, and that the old red blood cells did not increase in their ergothioneine content, then variations in the blood ergothioneine levels in the above experiment may be explained on several factors.

a. If reticulocytes are produced that have the same concentration of ergothioneine per cell as the average in the circulating erythrocytes, then since the reticulocytes are larger than the erythrocytes, for the same volume of corpuscles, the blood richer in reticulocytes will have a smaller concentration of ergothioneine. This effect would tend to decrease the ergothioneine content of the blood of the rats that have been bled frequently and it makes the

results obtained above all the more significant.

b. If reticulocytes are produced that have a greater content of ergothioneine per cell than the average amount in the circulating erythrocytes, then as the ratio of reticulocytes to erythrocytes increases, the ergothioneine level will increase. Rats 2, 5, 4, and 6 could show this effect because the source of ergothioneine to the rat was increased through injection. However, rats 1 and 3 can be assumed to have a more or less constant source of ergothioneine arising from their stock diet. Since their blood level increased after exsanguination, it suggests the possibility that, normally, new cells may be produced that contain more ergothioneine than is present in the old cells. This means, then, that as the cell grows older its ergothioneine content decreases. Perhaps in this way, ergothioneine performs a function in the red blood cell which causes its concentration to decrease.

This experiment should open up new pathways for future research on this subject. Ergothioneine itself can be used to study the mechanism whereby this compound is incorporated into the red cell. With the chemical synthesis of this compound, ergothioneine with labeled elements should be available for a more complete study of its role in the organism.

SUMMARY

1. Ingestion of ergothioneine can result in increases in blood ergothioneine in the rat.
2. Ingested ergothioneine was not excreted in any detectable form in urine or feces, as tested by our methods.
3. A method of extraction of ergothioneine from liver tissue has been described. In the rat, ergothioneine was found to be more concentrated in the liver than in the corpuscles, based on a dry weight basis.
4. Choline has no effect in increasing blood ergothioneine when it is fed with a casein diet.
5. Histidine supplement to a low protein barley diet was found to have no effect on the blood ergothioneine level.
6. Low protein barley diets resulted in a general decrease in the blood ergothioneine level in rats.
7. Methionine had no effect in increasing the blood ergothioneine when it was fed with a 1:1 mixture of Purina and Victor Fox checkers.
8. Diets with increased percentage of casein showed increased effectiveness in lowering the blood ergothioneine levels in weanling rats.
9. Dried blood supplements added to a 10% casein diet were effective in increasing blood ergothioneine, probably because of the ergothioneine content in the blood.
10. Purina Fox checkers caused increases in blood ergothioneine. The factor in the diet could be removed by extracting with

either one of hot water or ether.

12. Injected ergothioneine was completely removed from th e plasma within an hour of the injection.
13. Nephrectomized rats injected with ergothioneine were not able to completely remove the ergothioneine from the plasma within 2 hours. About 10% of the injected ergothioneine remained circulating in the plasma.
14. Injected ergothioneine caused increases in the blood ergothioneine level about 3 or 4 days after the injection.
15. Exsanguination of rats which have ergothioneine present in the blood, caused increases in the blood ergothioneine.

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